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13. ABSTRACT (Maximum 200 words)

Cells employ sensor biomolecules to dynamically evaluate their environments and trigger appropriate metabolic responses. The ability to program cells with engineered molecules that sense structural and chemical events and translate these events to controlled cellular behavior is a critical technology for challenges present in medical research and biotechnology. Recent studies have demonstrated the prevalence and diversity of nucleic acids that function as sensors and regulators of gene expression. Recent efforts in nucleic acid engineering have succeeded in the generation of synthetic nucleic acid molecules that regulate gene expression through diverse mechanisms. The goal of this proposal is to develop a platform for the design of nucleic acid molecules that will program and control targeted cellular behavior. Specific aims include to: (i) Design ligand-controlled RNA switches that regulate gene expression in mammalian systems; (ii) Construct engineered RNA switches that program cellular fates; (iii) Construct RNA switches that sense and respond to endogenous signals; (iv) Design RNA switches that detect viral infections in model systems; (v) Apply engineered RNA switches to the inhibition of expression of key viral and host proteins. The long-term objective is to develop enabling tools for programming cellular response for applications in intelligent therapeutics and metabolic reprogramming.

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Enclosure 1

Intelligent therapeutics and metabolic programming through tailor-made, ligand-controlled RNA switches

Brief report on progress towards the project's specific aims:

1. Design of ligand-controlled RNA switches for mammalian systems

A. Design of a small molecule-controlled switch platform

Over the past year we have demonstrated several different microswitch platforms that are functional in a variety of cell lines. Three regulatory mechanisms have been examined in our laboratory: antisense inhibition, ribozyme cleavage, and RNA interference (RNAi)-mediated gene silencing. Antisense-based platforms, similar to the antiswitch platform previously developed in our laboratory with demonstrated activity in *Saccharomyces cerevisiae*, demonstrated no significant knockdown of reporter gene expression (green fluorescent protein, GFP) in HEK293T and HeLa cell lines. However, the latter two regulatory mechanisms, ribozyme-based inactivation and RNAi-mediated silencing, demonstrated significant activity in these cell lines as is briefly described below.

Microswitches responsive to the small molecule theophylline and targeting GFP based on a ribozyme cleavage mechanism were constructed and examined in HeLa cell lines. These platforms demonstrate theophylline-regulated inactivation of gene expression *in vivo* (Figure 1). As illustrated, an 'ON' microswitch was constructed and characterized that down-regulates GFP levels in the absence of theophylline (by inactivating the target mRNA through catalytic cleavage) and up-regulates GFP levels in the presence of theophylline (as theophylline binding to this microswitch disrupts the catalytic core of this molecule).

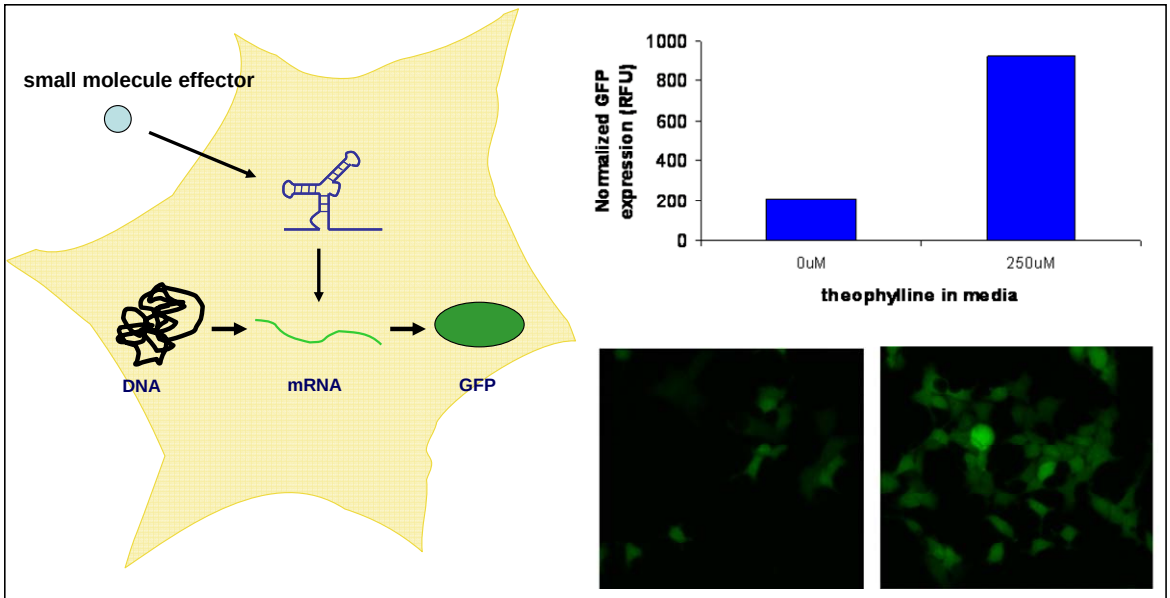

Figure 1. Theophylline-dependent regulation of GFP by a ribozyme-based microswitch platform. The microswitch harbors a ribozyme domain that is inactive in the presence of theophylline.

We have also made significant progress over this year in developing small molecule-responsive microswitches that act through the RNAi pathway. We have made progress on testing microswitches that function through three RNAi substrates: small interfering RNAs (siRNAs), small hairpin RNAs (shRNAs), and naturally-occurring microRNAs (miRNAs). Platforms based on regulating siRNA or shRNA activity are responsive to cytoplasmic ligands, whereas those based on regulating miRNA activity are responsive to nuclear ligands. Control experiments conducted earlier this year demonstrated that these molecules cannot be robustly synthesized with T7-based transcription mechanisms for exogenous delivery, due to the promiscuity of this polymerase and the difficulty in removing 5' phosphates completely for *in vivo* applications. Therefore, our experiments currently focus on expressing these molecules in the cell from different plasmid constructs.

The activity of theophylline-responsive microswitch platforms that function through regulating the activity of a shRNA substrate have been demonstrated. These platform designs utilize the conformational change induced by ligand-binding to these switch molecules to regulate Dicer processing. Designs displaying both 'ON' and 'OFF' behavior have been constructed (Figure 2). Microswitch platforms targeting a destabilized GFP have been characterized in HEK293T cells expressing each of the platforms from a U6 promoter on a plasmid construct. Fluorescence of transfected cells was analyzed by fluorescence microscopy and measuring the fluorescence of the cell lysate in a fluorescent plate reader. We have demonstrated that control shRNAs with different internal bulges are effective at silencing gene expression (Figure 3A, C). The shRNA control (sh3) was subsequently used as the base structure for microswitch designs. Two initial theophylline-regulated microswitch designs were constructed and characterized for their ability to induce theophylline-regulated RNAi-mediated silencing of GFP (Figure 3B, D). Both microswitches yielded 'ON' behavior with significant theophylline dependence as compared to control constructs. Experiments conducted across a range of theophylline concentrations indicate that ON1 displays a ramped dependence on theophylline levels and does not appear to saturate at the highest concentration

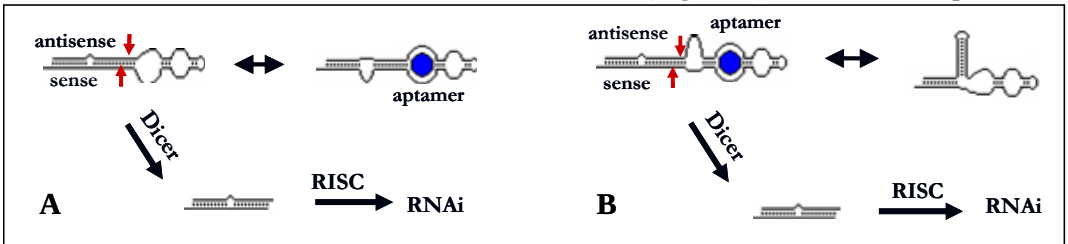

Figure 2. Design schemes for ligand-regulated shRNAs that rely on the requirement for a fully-formed stem for efficient Dicer cleavage. **A.** Designs exhibiting 'ON' behavior as designated by the expression level of the target gene in the presence of the ligand. **B.** Designs exhibiting 'OFF' behavior. The presence of the ligand stabilizes the conformation with a formed aptamer. Red arrows indicate Dicer cleavage sites.

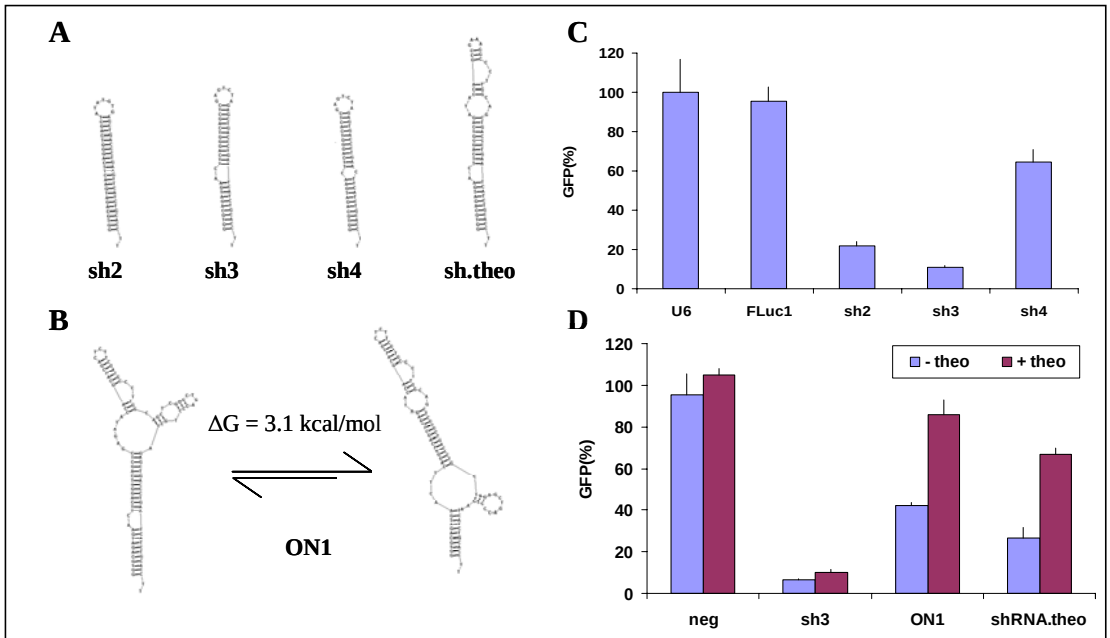

Figure 3. Theophylline-regulated shRNAs endogenously expressed from U6 promoters. **A.** Secondary structures of the shRNA controls. **B.** Theophylline-regulated shRNA exhibiting 'ON' behavior with the disfavored conformation containing the formed theophylline aptamer (right). **C.** A shRNA resembling pre-miR-30 (sh3) produces potent knockdown of GFP equivalent to the original shRNA (sh2) and better than a shRNA with a single mismatch in the sense strand (sh4). U6: vector with U6 promoter controlling expression of a scrambled transcript; FLuc1: U6-expressed miRNA targeting firefly luciferase. **D.** Theophylline-dependent 'ON' activity of two shRNA constructs ON1 and a shRNA with the theophylline aptamer in place of the loop (sh.theo). Cells were grown in the presence and absence of 1 mM theophylline. Error bars represent three independent measurements.

tested (1 mM) (Figure 4), suggesting that a greater dynamic range of GFP levels may be obtained by using higher theophylline concentrations or higher affinity aptamers.

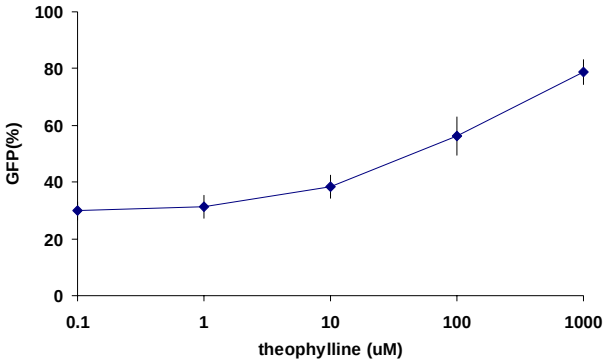

Figure 4. Theophylline-dependent knockdown of GFP by ON1. Error bars represent three independent measurements.

The activity of theophylline-responsive microswitch platforms that function through regulating the activity of a miRNA substrate have also been demonstrated. Platform designs for ligand-regulated miRNAs were based on structural requirements for efficient Drosha processing (Figure 5A-D). Initial tests with ligand-regulated miRNAs were conducted by expressing miRNAs targeting GFP from the 3' untranslated region (UTR) of DsRed-Express. Since Drosha cleavage separates the transcript from the polyA tail, a single transcript will either be processed by Drosha to activate RNAi-mediated silencing or exported to the cytosol for translation. Measuring differences in DsRed-Express levels provides insight into the extent of Drosha processing. Plasmids harboring DsRed-Express and a GFP-targeting miRNA in the 3' UTR under the control of a CMV promoter were transfected into HEK293T cells expressing a destabilized GFP and assayed for fluorescence as described above. Two theophylline-regulated designs (Figure 6A) were characterized for their ability to induce theophylline-dependent knockdown of GFP. miR_Sw1 demonstrated 'ON' behavior with modest theophylline dependence, whereas miR_Sw2 also demonstrated 'ON' behavior with minimal theophylline dependence (Figure 6B). DsRed-Express levels from these constructs demonstrate different extents of theophylline-dependent processing by Drosha (Figure 6C).

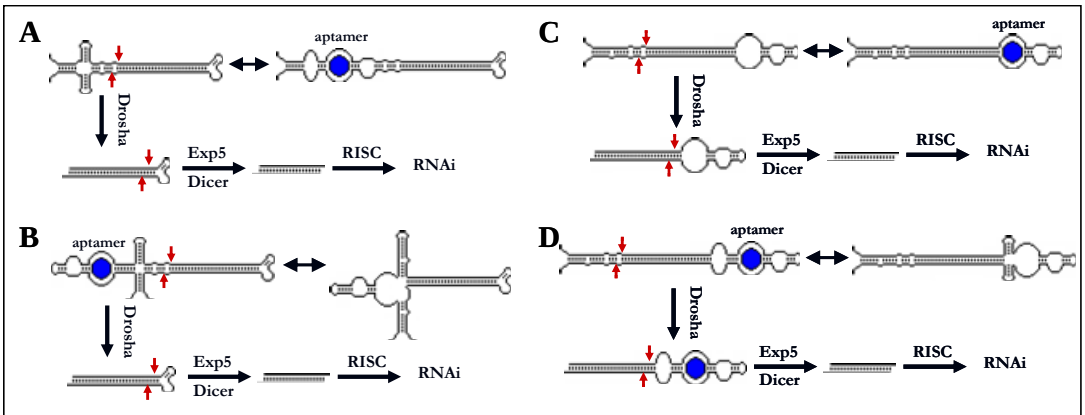

Figure 5. Design schemes for ligand-regulated miRNAs with aptamers inserted into the loop or stem region. **A.** Design exhibiting 'ON' behavior with aptamer located in stem. **B.** 'OFF' behavior with aptamer located in stem **C.** 'ON' behavior with aptamer located in loop. **D.** 'OFF' behavior with aptamer located in loop. Red arrows designate cleavage sites. Exp5: Exportin-5.

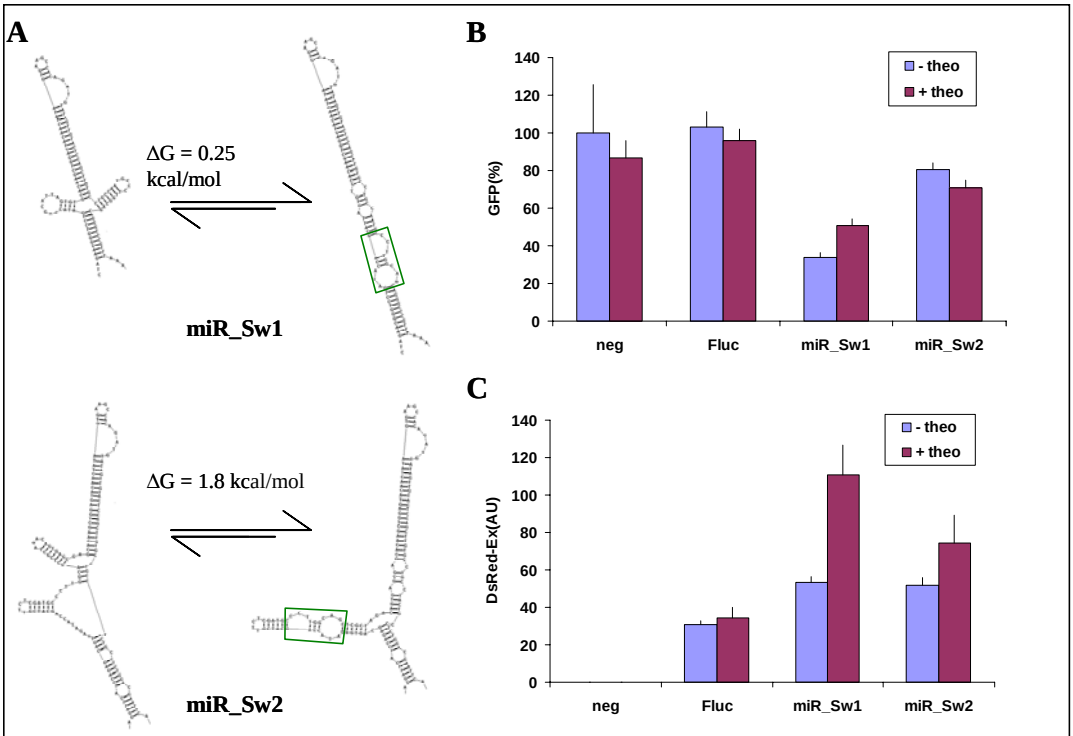

Figure 6. Theophylline-regulated miRNAs display limited responsiveness. **A.** Secondary structures and relative free energies for two regulated miRNAs (miR_Sw1 and miR_Sw2) designed to display 'OFF' behavior through stabilization of the disfavored conformation with a formed theophylline aptamer (green). Both miRNAs targeting GFP were inserted into the 3'UTR of DsRed-Express in a plasmid construct and transfected into HEK293T cells in the presence (+theo) or absence (-theo) of 1 mM theophylline. **B.** Fluorescence measurement of GFP levels. **C.** Fluorescence measurement of DsRed-Express. Error bars represent three independent measurements. neg: untransfected cells; Fluc: miRNA targeting Firefly Luciferase harbored in the 3'UTR of DsRed-Express; AU: arbitrary units.

These microswitch platforms are currently being explored for their modularity, tunability, and dynamic range. The work conducted over the past year in this laboratory has developed different small molecule-controlled microswitch platforms and demonstrated activity in different cell culture lines.

B. Design of a protein-controlled switch platform

Current work is exploring the construction of protein-responsive switches in the RNAi-based microswitch platform described above. Expression constructs have been made to express the Rev peptide and a Rev-DsRed-Express fusion construct in cell culture. Earlier experiments with the full-length Rev protein conducted in our laboratory demonstrated that the Rev protein affects expression levels of proteins (independent of protein-coding sequence) from a CMV promoter (which is used to express reporter genes in our cell lines). However, the Rev peptide does not have this affect on expression levels from the CMV promoter and will be used in subsequent studies. The Rev fusion construct indicated above will be used to monitor localization of this peptide fusion in later studies planned in our laboratory. Microswitch platforms responsive to the Rev peptide have been constructed and are currently being characterized in HEK293T and HeLa cell lines.

As will be described later in this report, we have demonstrated that the ribozyme-based microswitch platform can be used *in vivo* to detect endogenous protein ligand inputs. This data demonstrate that steric factors associated with binding larger ligands do not impede the action of these switch molecules.

2. Application of RNA switches to programming cellular fate

A. Programming apoptosis with microswitches

We have demonstrated that in addition to regulating the expression of heterologous genes such as fluorescent reporter proteins, microswitches can also regulate the expression of endogenous gene targets. Specifically, we have constructed microswitches that target a number of different anti-apoptotic gene targets. Based on assaying cell death through morphology, tryphan blue counting, and various commercially available caspase kits, the microswitches targeting the anti-apoptotic gene bcl-2 were determined to be most effective in inducing cell death in response to the presence of their input ligands in both HeLa and HEK293T cell lines (Figure 7).

B. Programming metabolic responses with microswitches

Progress on milestones associated with this specific aim is targeted for after the first year.

C. Programming immune responses with microswitches

Progress on milestones associated with this specific aim is targeted for after the first year.

3. Applications of RNA switches to sensing endogenous signals or markers

A. High-throughput, parallel selection of sensing domains

Significant progress has been made on establishing high-throughput, rapid selection schemes for small molecule and protein aptamers in my laboratory over the past year. We have successfully demonstrated the selection of a protein aptamer using capillary electrophoresis-based partitioning schemes (data not shown). One limitation of this partitioning method, is that it is very challenging, if not impossible, to use it for the selection of small molecule aptamers. This is because partitioning is based on changes in electrophoretic mobility, or mass to charge (m/z) ratios, and the binding of a small ligand such as a small molecule does not change the m/z ratio significantly enough to achieve effective partitioning of bound from unbound pools. To overcome this limitation, we are currently developing methods for the direct selection of microswitches responsive to small molecule and protein inputs. Using the microswitch platforms, we are developing general methods that will induce a significant change in the m/z ratio of the switch platform when bound to both small molecule and protein ligands. In addition, we have developed high-throughput, precise characterization assays for aptamers and RNA switches based on surface plasmon resonance analysis (data not shown). This technology will enable us to rapidly and robustly select and characterize switches to desired inputs for which aptamers do not currently exist. They will also enable us to select sensor domains that conform to the mechanism of the switch platform desired.

B. Sensing endogenous small molecule levels

Progress on milestones associated with this specific aim is targeted for after the first year.

C. Sensing endogenous protein levels

We have demonstrated the functional activity of a protein-responsive microswitch that responds to changes in the intracellular level of an endogenous protein. A microswitch that is able to detect the level of a phosphorylated mitogen-activated protein kinase, ERK-2, was constructed and characterized for activity in HeLa cell culture. The level of phosphorylated ERK-2 was regulated *in vivo* by the addition of phorbol 12-myristate 13-acetate (PMA), which has been demonstrated to increase the level of phosphorylated ERK-2 when added to cell culture. This microswitch was designed to regulate a cellular fate decision, specifically apoptosis, in the presence of elevated levels of phosphorylated ERK-2 (Figure 7). Specifically, this microswitch was designed with an input domain responsive to phosphorylated ERK-2, using a previously selected aptamer to this protein, and an output domain targeting the anti-apoptotic gene bcl-2. As illustrated, cells harboring the microswitch and elevated levels of phosphorylated ERK-2 exhibit significantly higher levels of cell death than all controls. Therefore, we have demonstrated that microswitches can sense endogenous protein levels (even at the level of changes in the post-translational modification state of a protein) and effectively regulate cellular behavior by targeting the expression levels of endogenous genes in response to activating biomolecular inputs. Current work is exploring the

concentration response functions of these switches and the modularity of these systems to different input and output domains.

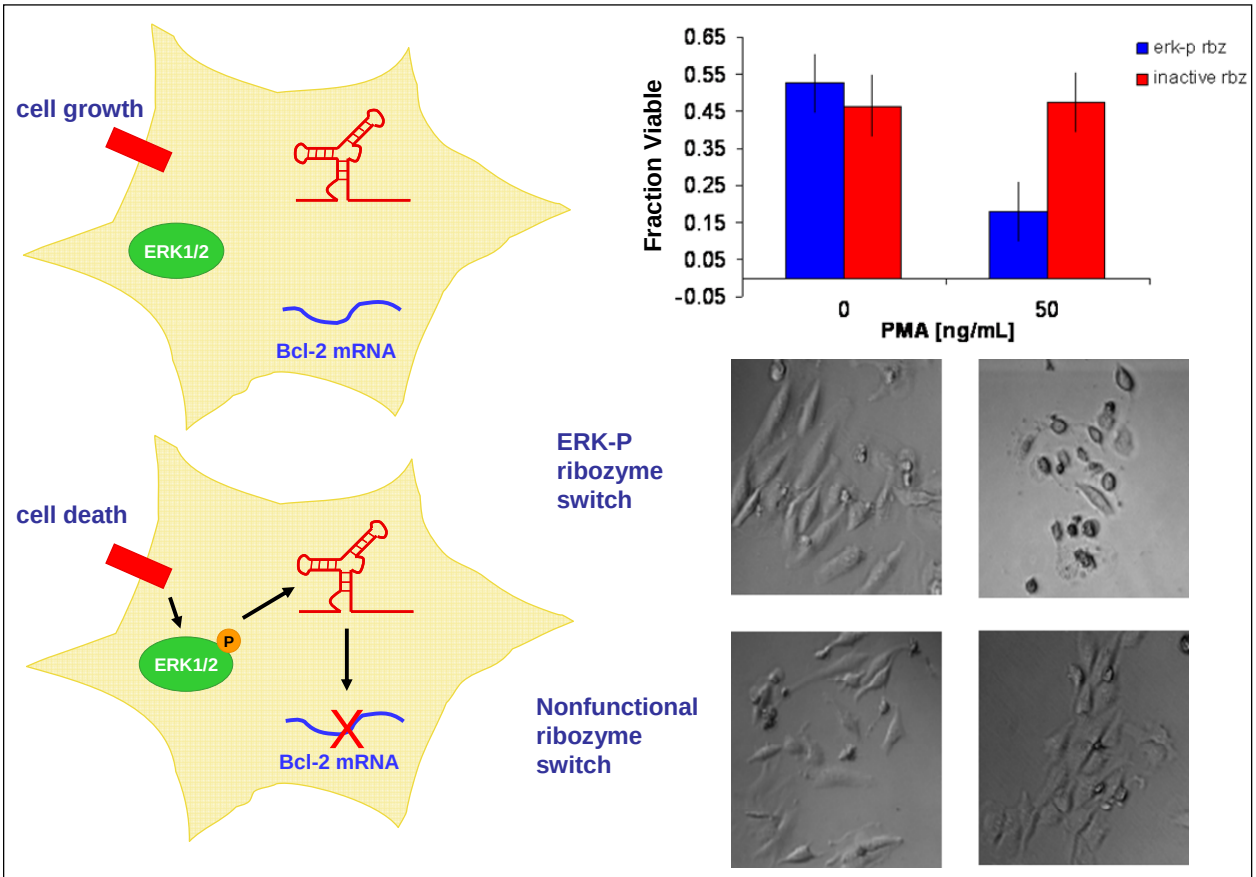

Figure 7. A protein-controlled microswitch platform that can respond to a protein-based disease biomarker and regulate the expression levels of an anti-apoptotic gene, bcl-2. This microswitch induces apoptosis when detecting increased levels of phosphorylated ERK-2, indicative of increased signaling through a growth signaling pathway.

D. Sensing disease or infectious biomarkers

The progress on sensing endogenous protein levels with the engineered microswitches (as described above in Section 3.C) supports the ability of these molecular switches to sense disease biomarkers. Increased levels of phosphorylated MAP kinases such as ERK2 are indicative of increased signaling through growth signaling pathways. In many examples activated signaling through these pathways is associated with the uncontrolled cell growth present in cancer. In addition, current on-going work is illustrating the ability of these molecules to detect biomarkers indicative of infectious disease such as different viral biomarkers associated with HIV and influenza.

4. Applications of RNA switches to sensing infectious disease in cell culture

Progress on milestones associated with the specific aims in this research thrust is targeted for after the first year.

5. Applications of RNA switches to targeting the expression of key viral and host proteins in infected cells

Progress on milestones associated with the specific aims in this research thrust is targeted for after the first year.